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HIV-Specific CAR T Cells with CD28 or 4-1BB Signaling Domains Are Phenotypically and Functionally Distinct and Effective at Suppressing HIV and Simian Immunodeficiency Virus

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ABSTRACT

Despite mounting a robust antiviral CD8 T cell response to HIV infection, most infected individuals are unable to control HIV viral load without antiretroviral therapy (ART). Chimeric Ag receptor (CAR) T cell treatment is under intensive investigation as an alternative therapy for ART-free remission of chronic HIV infection. However, achieving durable remission of HIV will require a successful balance between CAR T cell effector function and persistence. CAR T cells with CD28 costimulatory domains have robust effector function but limited persistence in vivo, whereas CAR T cells with 4-1BB costimulatory domains present a more undifferentiated phenotype and greater in vivo persistence. We compared the in vitro phenotype and function of rhesus macaque and human CAR T cells that contained either the CD28 or 4-1BB costimulatory domain; both constructs also included CARs that are bispecific for gp120 of HIV or SIV and the CXCR5 moiety to promote in vivo homing of CAR/CXCR5 T cells to B cell follicles. Cells were transduced using a gammaretroviral vector and evaluated using flow cytometry. 4-1BB-CAR/CXCR5 T cells were phenotypically distinct from CD28-CAR/CXCR5 T cells and showed increased expression of CAR and CD95. Importantly, both CD28- and 4-1BB-CAR/CXCR5 T cells retained equal capacity to recognize and suppress SIV in vitro. These studies provide new insights into rhesus macaque and human 4-1BB- and CD28-bearing CAR T cells.

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INTRODUCTION

Despite exhibiting a robust CD8 T cell response, the human immune system is unable to control HIV infection without intensive and sustained antiretroviral therapy (ART). Although ART has increased the lifespan of millions of individuals living with HIV, life-long ART treatment is burdensome and can be accompanied by serious side effects. Therefore, it is critical to develop alternative treatments for achieving durable remission of HIV infection without ART.

Chimeric Ag receptor (CAR) T cell therapy is an attractive approach for HIV treatment because the engineered receptor can specifically target conserved regions of HIV, bypass the restriction of the TCR and MHC interaction, and reduce the chance for immune-mediated viral escape. Some of the first CAR T cell clinical trials used CD4-based HIV-specific CARs (1, 2).

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Abbreviations used in this article: ART, antiretroviral therapy; BFA, brefeldin A; CAR, chimeric Ag receptor; CM, central memory; CRD, carbohydrate recognition domain; CTV, CellTrace Violet; EM, effector memory; Env, envelope; D, day; GRV, gammaretroviral; h, human; MBL, mannose-binding lectin; PSG, penicillin/streptomycin/glutamate; TM, transmembrane.

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However, despite promising in vitro activity (3, 4), these first-generation CAR T cells were unsuccessful at suppressing plasma viremia in vivo. Likewise, CAR T cells generated using PBMCs from SIV/HIV- and SIV-infected rhesus macaques showed antiviral activity in vitro, but limited persistence in vivo (5). A second-generation CD4-based CAR including a bispecific Ag recognition domain for the D1D2 domain of CD4 and the carbohydrate recognition domain (CRD) of mannose-binding lectin (MBL), to target gp120 of HIV envelope (Env), greatly improved the utility of CD4-based HIV CAR T cells (6). Dual specificity increases the likelihood of recognizing HIV Env and decreases the likelihood of escape mutants. Importantly, addition of the MBL motif removes the entry-receptor function of the CD4 motif (6). Recently, we showed that infusion of T cells expressing an SIV-specific CD4-MBL-CAR and the CXCR5 follicular homing receptor (CAR/CXCR5 T cells) could successfully target viral RNA+ cells in lymphoid follicles in SIV-infected rhesus macaques; however, the infused CAR/ CXCR5 T cells declined to undetectable levels after 2–4 wk (7). As these CAR/CXCR5 T cells were generated using a construct containing a CD28 costimulatory domain, we hypothesized that a different costimulatory domain might confer greater persistence of CAR/CXCR5 T cells.

Numerous studies have reported increased longevity and persistence of CAR T cells with 4-1BB costimulatory domains (8–12). Recent studies comparing CD28- and 4-1BB-CAR T cells in both animal model and human clinical trials show that while CD28-CAR T cells have an early advantage with increased effector function, 4-1BB-CAR T cells show greater persistence (13–15). One explanation for this increased persistence is a difference in cellular metabolism. CD28-CAR T cells primarily rely on glycolysis for energy, whereas 4-1BB-CAR T cells have increased mitochondrial biogenesis and oxidative phosphorylation, promoting longevity (16).

One concern of gammaretroviral (GRV) vector transduction is ligand-independent tonic signaling. Tonic signaling is a natural process of endogenous T cells necessary for cell survival and maintenance of peripheral T cell tolerance (17). Recently appreciated is the potential deleterious effect of tonic signaling of CAR T cells. Certain combinations of CAR, hinge, and costimulatory domains are prone to ligand-independent tonic signaling and T cell exhaustion. Tonic signaling in CD28-CAR T cells can be measured by prolonged expansion, constitutive cytokine release, and increased effector phenotype (18). However, tonic signaling in 4-1BB-containing CAR T cells has been characterized by increased apoptosis, rather than overexpansion (19, 20). Increased CAR expression is often a useful surrogate for tonic signaling (21). However, as tonic signaling has been reported in CAR T cells expressing both CD28 and 4-1BB signaling domains, it is important to determine the in vitro impact of tonic signaling in each CAR T cell system prior to launching in vivo studies (19, 21).

To investigate the potential for use of CD4-MBL-4-1BB/CAR/CXCR5 (4-1BB-CAR/CXCR5) T cells for the treatment of HIV/SIV infection, we examined the phenotype and in vitro functionality of 4-1BB-CAR/CXCR5 T cells compared with CD4-MBL-CD28/CAR/CXCR5 (CD28-CAR/CXCR5) T cells. To our knowledge, this study is the first comparison between CD28 and 4-1BB costimulatory domains during in vitro expansion, prior to stimulation through the ligand-binding domain of the CAR. Additionally, to our knowledge, this is the first comparison of CD28 and 4-1BB costimulatory domains in rhesus macaque and human PBMCs transduced with GRV containing the CAR/CXCR5 construct. In CAR T cells derived from rhesus macaques, we detected phenotypic differences between CD28- and 4-1BB-CAR/CXCR5 T cells, with 4-1BB-CAR/CXCR5 T cells showing increased surface expression of CD4-MBL, Fas (CD95), and HLA-DR after transduction. Despite this increase in activation, 4-1BB-CAR/CXCR5 T cells maintained a more central memory (CM)–like phenotype than CD28-CAR/CXCR5 T cells. Whereas 4-1BB-CAR/CXCR5 T cells showed lower levels of effector cytokines, both CD28- and 4-1BB-CAR/CXCR5 T cells retained equal capacity to recognize and suppress SIV and HIV in vitro. In CAR T cells derived from human PBMCs, we found increased apoptosis and decreased expansion in 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR T cells during primary expansion. However, both CAR T cells displayed similar phenotypes after primary expansion in vitro. Importantly, both human (h)4-1BB-CAR/CXCR T cells and hCD28-CAR/CXCR5 T cells suppress HIV in vitro.

These data suggest that increased CAR expression and activation does not lead to CAR T cell dysfunction in CD4-MBL-CAR/CXCR5 T cells in rhesus macaque or human PBMCs in vitro and provide a rationale for future studies using 4-1BB-CAR/CXCR5 T cells as treatment for HIV/SIV in vivo.

**MATERIALS AND METHODS**

**Cells**

Rhesus PBMCs were obtained from animals housed at the Wisconsin National Primate Research Center. The cells were collected by density gradient centrifugation, cryopreserved in CryoStor CS5 (BioLife Solutions) at a concentration between 5 and 20 million cells/ml, and transported and stored in liquid nitrogen until use. Human PBMCs were isolated by density gradient centrifugation from Trima Cones obtained from Innovative Blood Resources. Cells were cryopreserved at a density of 10–20 million cells/ml in 90% FBS/10% DMSO and stored in liquid nitrogen.

**Virus production**

GRVs were produced by Lipofectamine-mediated transfection of 293T cells cotransfected with pBS-CMV-gagpol, RDI14, and pMD.g, as described previously (22). The CD4-MBL CAR/CXCR5 construct was described previously. Briefly, the bispecific CAR contains rhesus CD4 and MBL domains specific for SIV, linked to either a CD28 hinge, transmembrane (TM) and costimulatory domain (6, 22, 23), or a CD8 hinge and TM domain followed by a 4-1BB costimulatory domain (24). Both

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constructs use a CD3ζ signaling domain. The follicular homing receptor, CXCR5, is linked to the CAR with a self-cleaving peptide, P2A with a GSG linker at the N terminus (25). The human constructs, which are specific for HIV, contain human sequences for the same elements. Sequences for all constructs are provided in Supplemental Fig. 1.

Transduction
Rhesus macaque PBMCs were transduced as described previously (26). Human PBMCs were transduced using identical methods, with the modification of using anti-human CD3 (clone OKT3 obtained from the National Cancer Institute’s Biological Resources Branch Preclinical Repository) (7, 26).

Immunophenotyping by flow cytometry
Multiparametric flow cytometry was used to characterize surface and intracellular protein expression. Abs used in flow cytometry are described in Supplemental Table I. All samples were acquired on a Beckman Coulter CytoFLEX. A minimum of 100,000 events were acquired for each sample. Data were analyzed with FlowJo v10 (Becton Dickinson).

Ag-specific stimulation for rhesus CAR/CXCR5 T cells
On day (D)9 of transduction, autologous SIV+ or SIV− PBMCs were thawed, depleted of CD8+ T cells, and labeled with CellTrace Violet (CTV, Thermo Fisher Scientific/Invitrogen). A total of 5 × 10^5 CD8-depleted, CTV-labeled PBMCs were incubated with SIVmac239 gp130 (National Institutes of Health AIDS Reagent Program) at a dose of 1 μg/3.5 × 10^5 cells in 100 μL of X-VIVO 15, 10% FBS, 1% penicillin/streptomycin/glutamate (PSG), and 50 IU/ml IL-2 for at least 20 min before adding CD28-, 4-1BB-, or mock-transduced cells. After 1 h of coculture, 1× brefeldin A (BFA) (BioLegend) and anti-human CD107a Brilliant Violet 786 (BioLegend, clone H4A3) were added, and cultures were incubated overnight (14–18 h) at 37°C. After overnight stimulation, cells were washed and stained for extracellular and intracellular markers as described in Supplemental Table I.

SIV suppression assay
To generate target cells for the SIV suppression assay, SIV+ PBMCs from autologous animals were enriched for CD4+ T cells (Miltenyi Biotec nonhuman primate CD4+ enrichment kit, catalog no. 130-092-144) and stimulated with PMA (50 ng/ml), ionomycin (5 μg/ml), and 100 IU/ml IL-2 in RPMI 1640, 10% FBS, and 1% PSG for 2 d. On D2 of stimulation, cells were washed and replated in fresh media containing 100 IU/ml IL-2. Fresh media were added on D4, and cells were plated for assay on D7. Prior to plating on D7, cells were labeled with CTV according to the manufacturer’s protocol (Thermo Fisher Scientific/Invitrogen). Freshly transduced cells were added on D9 of stimulation and incubated for 48–72 h at 37°C. After culture, cells were stained with Live/Dead Near-IR (Thermo Fisher Scientific/Invitrogen). Cells were fixed and permeabilized using BD Cytofix/Cytoperm and stained intracellularly with SIV gag-p27 (NIH AIDS Reagent Program) Alexa Fluor 488 (conjugated with an Alexa Fluor 488 labeling kit, Thermo Fisher Scientific, catalog no. A20181). Samples were acquired on a Beckman Coulter CytoFLEX and analyzed using FlowJo 10.7.

HIV suppression and cytotoxicity assay
8E5 cells were kept at a low passage number (<10 passages) and labeled with CTV. 8E5 cells (target) were cocultured with either CD28-, 4-1BB-, or mock-transduced cells (effector) at a 1:2 E:T ratio for 48 h at 37°C in X-VIVO 15 media supplemented with 10% FBS, 1% PSG, and 50 IU/ml IL-2. Cells were harvested and stained with Live/Dead Near-IR (Thermo Fisher Scientific/Invitrogen). Cells were fixed and permeabilized using BD Cytofix/Cytoperm and stained intracellularly for HIV gag (anti-p24, KC57-PE conjugate, obtained through the NIH HIV Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health; anti–HIV-1 p24 mAb, ARP-13449, contributed by Division of AIDS/National Institute of Allergy and Infectious Diseases). To determine cytotoxic activity, the frequency of live CTV+ target cells remaining in culture was compared with the frequency of live CTV+ target cells in mock culture. Samples were acquired on a Beckman Coulter CytoFLEX and analyzed using FlowJo 10.7.

Migration assay
Transduced rhesus macaque PBMCs (1 million cells in 100 μL of X-VIVO 15 containing 0.1% BSA) were placed in the upper chamber of a Transwell plate (Costar) with a 5.0-μm membrane. The lower chamber contained X-VIVO/1% BSA only or X-VIVO/1% BSA containing the chemokine CXCL13 (2.5 μg/ml) or the positive control, CXCL12 (1 μg/ml). After incubating for 4 h at 37°C, cells were collected and counted on a CytoFLEX flow cytometer (Beckman Coulter). All samples were normalized with the addition of AccuCheck counting beads (Thermo Fisher Scientific). Specific migration was determined by subtracting the number of cells that migrated to X-VIVO/BSA alone from the number that migrated to the chemokine and then dividing by the number of input cells.

Statistical analysis
All statistical analyses were performed using GraphPad Prism v8.
RESULTS

Rhesus macaque PBMCs transduced with GRV containing either CD28- or 4-1BB-CAR/CXCR5 are capable of robust expansion in vitro

To determine the feasibility of using 4-1BB-CAR/CXCR5 T cells in vivo, we investigated the transduction efficiency and expansion potential of 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR/CXCR5 T cells in rhesus macaque PBMCs. As shown in Fig. 1A, both CAR constructs contain the D1D2 region of CD4, which targets the CD4-binding site of SIV Env, and the CRD of MBL (CD4-MBL), the signaling domain of CD3ζ, and the B cell follicle homing molecule CXCR5. The key differences in these constructs occur in the TM and costimulatory domains. CD28-CAR/CXCR5 contains the CD28 TM and signaling domains, whereas 4-1BB-CAR/CXCR5 contains the CD8α TM domain and 4-1BB signaling domain. Flow cytometry analysis of transduced cells costained with anti-CD4 and anti-MBL Abs showed that 4-1BB-CAR/CXCR5 efficiently transduced rhesus PBMCs (Fig. 1B). Consistent with previous work (22, 26), we found that CXCR5 expression positively correlates with MBL expression (Supplemental Fig. 2A) and both CD28- and 4-1BB-CAR/CXCR5 T cells migrate toward CXCL13 at a greater frequency than mock-transduced cells (Supplemental Fig. 2B). At the end of primary expansion (D9 of culture), >80% of CD28- and 4-1BB-CAR/CXCR5 T cells were Ki67+, indicating robust expansion (Fig. 1D). However, compared with CD28-CAR/CXCR5 T cells, 4-1BB-CAR/CXCR5 T cells showed lower fold expansion in 10 of 13 paired samples (median = 11.30 and 10.09, p = 0.06, Fig. 1C) and a reduced frequency of cells undergoing apoptosis as measured by the percentage of annexin V+ cells (Fig. 1E). Taken together, these data suggest that rhesus macaque PBMCs can be efficiently transduced to generate 4-1BB-CAR/CXCR5 T cells that undergo proliferation and expansion in vitro.

Rhesus 4-1BB-CAR/CXCR5 T cells are phenotypically distinct from CD28-CAR/CXCR5 T cells

We used flow cytometry to further investigate the phenotype of CD28- and 4-1BB-CAR/CXCR5 T cells in rhesus macaque PBMCs after primary expansion. Full characterization of the cell product prior to infusion on D9 may lend insight into the correlates of success of CAR T cell therapy. In this study, we detected no significant difference in the frequency of CM (CD95–CCR7–CD28+) or effector memory (EM; CD95+ CCR7– CD28+) T cells (Fig. 2A). However, the frequency of transitional memory T cells differed significantly based on expression of the lymph node homing receptor CCR7; 4-1BB-CAR/CXCR5 T cells were enriched for CD28–CCR7+ transitional memory T cells, and CD28-CAR/CXCR5 T cells were enriched for CD28+ CCR7– T cells (Fig. 2A). CD28–CCR7+ T cells have been previously described as TEM1 cells (27). We detected a small but significant increase in activated 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR/CXCR5 T cells, measured by both CD25 (4-1BB median = 50.85%, CD28 median = 48.45%) and HLA-DR (4-1BB median = 45.10%, CD28 median = 17.35%) (Fig. 2B, 2C). Both CD28- and 4-1BB-CAR/CXCR5 T cells showed similar levels of PD-1 expression, with 4-1BB-CAR/CXCR5 T cells trending lower (median = 10.12 and 6.045%, respectively, Fig. 2F). We also detected an increase in relative CAR expression on 4-1BB-CAR/CXCR5 T cells (median = 63.78) compared with CD28-CAR/CXCR5 T cells (median = 36.24) (Fig. 2E). Increased CAR expression is often an indicator of tonic signaling (19, 28). Consistent with the more activated phenotype of 4-1BB-CAR/CXCR5 T cells, we found a small but significant increase in relative CD95 expression (with respect to mock-transduced cells) on the surface of 4-1BB-CAR/CXCR5 T cells (median = 1.575) compared with CD28-CAR/CXCR5 T cells.
cells (median = 1.055) (Fig. 2F). Taken together, these data are suggestive of differential activation after transduction and primary expansion in 4-1BB-CAR/CXCR5 T cells in rhesus macaque PBMCs.

**Decreased effector cytokine production but no change in SIV suppression in 4-1BB-CAR/CXCR5 T cells in vitro**

To further characterize CD28- and 4-1BB-CAR/CXCR5 T cells, we assessed the effector cytokine production and SIV suppression capacity of both populations. Consistent with previous reports of cytokine production in CD28- and 4-1BB-containing CAR T cells (11, 16, 29, 30), we found that CD28-CAR/CXCR5 T cells expressed greater levels of CD107a (median = 514.190 and 4.940%) and TNF (median = 20.533 and 8.905%) than 4-1BB-CAR/CXCR5 T cells after Ag-specific stimulation with SIV gp130 (Fig. 3A). We found minimal production of IFN-γ and IL-2 under these stimulation conditions (Fig. 3A). We compared the ability of CD28- and 4-1BB-CAR/CXCR5 T cells to suppress SIV in vitro. Autologous SIV-infected CD4+ T cells were measured by intracellular SIV gag p27 staining. Coculturing of SIV+
target cells with either CD28- or 4-1BB-CAR/CXCR5 T cells led to a significant reduction in p27\(^\text{\textsubscript{1}}\) in target cells compared with those cultured with mock-transduced cells. After 48 h, with an E:T ratio of 1:1, we found both CD28- and 4-1BB-CAR/CXCR5 T cells capable of suppressing SIV in vitro (Fig. 3B, 3C). These data suggest that although cytokine production is reduced in 4-1BB-CAR/CXCR5 T cells, they retain equal capacity to recognize and suppress SIV in vitro.

**Phenotypic characterisation of rhesus CAR T cells after primary expansion**

We previously observed an increase in CD95 expression on 4-1BB-CAR/CXCR5 T cells (Fig. 2E). To determine whether this increased CD95 had functional relevance in longer-term culture, we investigated the viability and fold expansion of 4-1BB-CAR/CXCR5 T cells expanded from D9 to D15. As shown in Fig. 4A, we found no difference in viability (measured by trypan blue) between CD28- and 4-1BB-CAR/CXCR5 T cells at D9 or D15. Although there were no statistically significant differences in fold expansion (Fig. 4B), apoptosis (annexin V, Fig. 4C), or proliferation (Ki67, Fig. 4D), each marker trended higher in the 4-1BB-CAR/CXCR5 T cells than in the CD28-CAR/CXCR5 T cells. Specifically, although we detected no statistically significant increase in apoptosis of 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR/CXCR5 T cells or mock-transduced cells, the median frequency of annexin V\(^+\) cells in 4-1BB-CAR/CXCR5 T cell culture was 9.30, compared with 6.36 for CD28-CAR/CXCR5 T cells and 4.41 for mock-transduced cells (Fig. 4C). These data are suggestive of increased cell death and proliferation in D15 culture in 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR/CXCR5 T cells. The frequency of PD-1\(^+\) cells was lower in both CD28- and 4-1BB-CAR/CXCR5 T cells compared with mock (Fig. 4F). These data suggest that increased cell culture time leads to increased proliferation and initiation of cell death, but no change in exhaustion marker PD-1 in 4-1BB-CAR/CXCR5 T cells compared with CD28-CAR/CXCR5 T cells.

**Human 4-1BB-CAR/CXCR5 T cells experience increased apoptosis and decreased expansion compared with CD28-CAR T cells during primary expansion**

To determine whether these results were species specific, we conducted the same transduction protocol and phenotypic and functional analyses in PBMCs from six human donors. h4-1BB-CAR/CXCR5 T cells showed a significant reduction in expansion compared with hCD28-CAR/CXCR5 T cells during primary expansion from D5 to D9 (Fig. 5A), although h4-1BB-CAR/CXCR5 T cells showed a robust level (~10-fold) of expansion, similar to the fold expansion in rhesus macaque (Fig. 1C). We detected similar frequency of proliferation, measured by Ki67, in h4-1BB- and hCD28-CAR/CXCR5 T cells (Fig. 5B). This discrepancy between frequency of proliferation and fold expansion is possibly explained by an increased frequency of h4-1BB-CAR/CXCR5 T cells undergoing apoptosis compared with hCD28-CAR/CXCR5 T cells at D9 (Fig. 5C). Consistent with our findings in rhesus macaque, we found that h4-1BB-CAR/CXCR5 T cells have significantly more CD45RO\(^+\)CD27\(^-\)CCR7\(^-\)TM phenotype cells compared with hCD28-CAR/CXCR5 T cells. We also detected increased frequency of CD45RO\(^+\)CD27\(^-\)CCR7\(^-\)CM cells in h4-1BB-CAR/CXCR5 T cells (\(p = 0.06\)) (Fig. 5D). We found increased frequency of HLA-DR\(^+\) 4-1BB-CAR/CXCR5 T cells (Fig. 5E) and no difference in coinhibitor expression (Fig. 5F), consistent with the phenotype observed in transduced rhesus macaque PBMCs. h4-1BB-CAR/CXCR T cells also showed increased expression of CD4-MBL-CAR (Fig. 5G) and CD95 (Fig. 5H), consistent with data from rhesus macaque PBMCs. Overall, rhesus and human CD28- and 4-1BB-CAR/
CXCR T cells showed similar phenotypes after primary expansion in vitro.

**Both h4-1BB-CAR/CXCR T cells and hCD28-CAR/CXCR5 T cells suppress HIV in vitro**

We investigated the in vitro functionality of h4-1BB-CAR/CXCR T cells by culturing them overnight in the presence of 8E5 cells expressing live attenuated HIV and assessing both effector cytokine production and cytotoxicity. Both hCD28- and h4-1BB-CAR/CXCR5 T cells showed robust production of CD107a and TNF in five out of six donors, with hCD28-CAR/CXCR5 T cells secreting more effector cytokines than h4-1BB-CAR/CXCR5 T cells (Fig. 6A). As with rhesus macaques, we were unable to detect robust IFN-γ or IL-2 production in these stimulation conditions. As a final determinant of in vitro function, hCD28- and h4-1BB-CAR/CXCR5 T cells, and mock-transduced cells were cocultured for 48 h with 8E5 cells. 8E5 cells were examined for expression of HIV gag p24 by flow cytometry. We detected no difference in the relative suppressive capacity of hCD28- and h4-1BB-CAR/CXCR5 T cells (Fig. 6B). Importantly, both hCD28- and h4-1BB-CAR/CXCR5 cultures showed a significant reduction in the frequency of HIV-infected, p24+ 8E5 cells compared with mock-transduced cultures (Fig. 6B).

To assess the cytolytic function of hCD28- and h4-1BB-CAR/CXCR5 T cells, we measured the frequency of live target (8E5) cells remaining after 48 h in culture. Compared to mock-transduced cells, both hCD28- and h4-1BB-CAR/CXCR5 T cells had reduced frequency of target cells, indicating cytotoxic function (Fig. 6C). As shown in Fig. 6C, there was no measurable difference in cytotoxicity of hCD28- and h4-1BB-CAR/CXCR5 T cells. Of note, we detected no decrease in frequency of live target cells when mock, hCD28-, and h4-1BB-CAR/CXCR5 T cells were cocultured with HIV-uninfected CEMx174 cells (Supplemental Fig. 3). Taken together, these data suggest that both CD28- and 4-1BB-CAR/CXCR5 T cells are effective at suppressing HIV and killing HIV-infected cells in vitro. These data are consistent with data in rhesus macaques and suggest that although h4-1BB-CAR/CXCR5 T cells may experience ligand-independent tonic signaling, it does not impact in vitro function.

**h4-1BB-CAR T cells after primary expansion**

To investigate the functional relevance of increased CD95 expression in h4-1BB-CAR/CXCR5 T cells, we followed cells from three donors in culture until D15. First, we examined the viability and number of hCD28- and h4-1BB-CAR/CXCR5 T cells at D9 and D15. At D9, there was no difference in median viability of any transduced cells (range, 93.5–92.5%); however, by D15,
the h4-1BB-CAR/CXCR5 T cells were all trending lower in viability (87%) than either hCD28-CAR/CXCR5-transduced (91%) or mock-transduced (92%) cells (Fig. 7A). In PBMCs from two of three donors, the frequency of apoptosis in h4-1BB-CAR/CXCR5 T cells was greater than in hCD28-CAR/CXCR5 T cells, but less than mock-transduced cells (Fig. 7B). Unexpectedly, there was no difference in fold expansion of h4-1BB-CAR/CXCR5 T cells compared with mock cells (2.4-fold) from D9 to D15, with a lower median expansion in hCD28-CAR/CXCR5 T cells (2.5-fold) (Fig. 7C). This finding suggests that after primary expansion, h4-1BB-CAR/CXCR5 T cells proliferate and are maintained at the same rate as hCD28-CAR/CXCR5- and mock-transduced cells in vitro. The frequency of proliferation was similar in mock, hCD28-, and h4-1BB-CAR/CXCR5 T cells, as measured by Ki67 level (Fig. 7D). hCD28-CAR/CXCR5 T cells had frequencies of two markers of cellular activation, CD25 and CD69, similar to mock cells, whereas two out of three donors showed h4-1BB-CAR/CXCR5 T cells with increased frequency of CD25, and three out of three donors showed increased CD69 compared with both mock and hCD28-CAR/CXCR5 T cells (Fig. 7D). These results suggest increased activation of h4-1BB-CAR/CXCR5 T cells at D15, consistent with D9 in rhesus macaques, excluding CD25 where we see a significant difference between CD28- and 4-1BB-CAR/CXCR5 T cells (Fig. 2). In transduced PBMCs from two out of three donors, hCD28- and h4-1BB-CAR/CXCR5 T cells showed decreased frequency

FIGURE 5. Human CD28- and 4-1BB-CAR/CXCR5 T cells are phenotypically and functionally distinct.

(A) Fold expansion of hCD28- and h4-1BB-CAR/CXCR5 T cells from D5 to D9. Fold expansion from D5 to D9 of culture was determined by dividing the number of live cells recovered on D9 by the number of cells added to the GREX on D5 (usually around 1 x 10^5 cells added on D5) (n = 6, Wilcoxon matched pairs rank test, *p < 0.05). Frequency of cells expressing (B) Ki67 and (C) Annexin V on D9 in culture (n = 6, Wilcoxon matched pairs rank test; ns = not significant, p < 0.05). (D) Frequency of hCD28- and h4-1BB-CAR/CXCR5 cells with indicated memory phenotype. All cells were previously gated on CD3^+CD4^+MBL^-. SC, CD45RO^+CD45RA^-CCR7^+CD27^-CD95^-; CD27^+TM, CD45RO^-CD45RA^+CCR7^-CD27^+; CM, CD45RO^-CD45RA^-CCR7^-CD27^-; CM, CD45RO^-CD45RA^-CCR7^-CD27^-; EM, CD45RO^-CD45RA^-CCR7^-CD27^-; CD27^+CD27^-CD95^+. Expression of (E) HLA-DR and (F) inhibitory markers PD-1 and TIM-3, by flow cytometry. (n = 6, Wilcoxon matched pairs rank test, ns = not significant, *p < 0.05). Example staining and comparison of geometric mean fluorescence intensity (gMFI) of (G) MBL and (H) CD95 on surface of mock- (open), CD28- (teal), and 4-1BB- (pink) transduced cells (n = 6, Wilcoxon matched pairs rank test, *p < 0.05). SCM, stem cell memory; TM, transitional memory.
of PD-1–expressing cells, compared with mock (Fig. 7G). However, both hCD28- and h4-1BB-CAR/CXCR5 T cells showed increased frequency of TIM-3, another T cell coinhibitory receptor commonly associated with T cell exhaustion (Fig. 7H). These data suggest that h4-1BB-CAR/CXCR5 T cells may have increased the likelihood of activation and coinhibitor expression by D15 of culture; however, a larger sample size will be required to fully characterize this phenotype.

**DISCUSSION**

This study investigated the in vitro effects of using the 4-1BB costimulatory domain in CD4-MBL-CAR/CXCR5 T cells. We used flow cytometry to assess the phenotype and function of HIV/SIV-specific CAR T cells containing either the CD28 or 4-1BB costimulatory domain in PBMCs from both rhesus macaques and humans and found similar characteristics in CAR T cells derived from rhesus macaques and human PBMCs. Both rhesus macaque and human 4-1BB-CAR/CXCR5 T cells were enriched for CCR7 and expressed lower levels of effector cytokines upon Ag stimulation but were equally able to suppress SIV/HIV in vitro. 4-1BB-CAR/CXCR5 T cells expressed more CAR and CD95 than did CD28-CAR/CXCR5 T cells. Despite an increase in CAR and CD95 expression, we detected similar levels of expansion of 4-1BB-CAR/CXCR5 T cells in rhesus macaques and human PBMCs (~10-fold). When cultures progressed to 15 d, we found similar expansion between mock, hCD28-, and h4-1BB-CAR/CXCR5 T cells, suggesting that increased CAR and CD95 expression does not contribute to decreased expansion in long-term culture of 4-1BB-CAR/CXCR5 T cells.

Overall, our data are consistent with previous reports of in vitro phenotype and function of CD28- and 4-1BB-containing CAR T cells. One of the primary reasons for examining different signaling domains is the well-established differences of in vivo persistence of CAR T cells with CD28 versus 4-1BB signaling domains (16, 31, 32). Recent reports suggest that a CD28-costimulatory domain promotes glycolytic metabolism and shorter-lived effector cells, whereas the 4-1BB-costimulatory domain promotes mitochondrial biogenesis and oxidative phosphorylation, leading to longer-lived, CM-like T cells (16). In our hands, we found increased frequency of CCR7-expressing cells in 4-1BB-transduced cells of both rhesus macaques and humans. This finding has important implications for treatment of HIV infection, where virus production is concentrated in lymph nodes. Increasing the frequency of CCR7+ cells by using the 4-1BB signaling domain combined with the CXCR5 motif in our construct could improve CAR T cell homing to lymph nodes and B cell follicles in general and lead to greater control of HIV/SIV.

Our results showed that 4-1BB-CAR/CXCR5 T cells secrete lower levels of cytokine than CD28-CAR/CXCR5 T cells in both rhesus macaque and human PBMCs. This finding is consistent with the well-established paradigm of cytokine secretion by different memory T cell subsets (33). Effector and EM T cells secrete cytokines more readily than do CM phenotype cells. As an additional consideration, our CD28 and 4-1BB constructs had different hinge and TM domains. It has previously been reported that CAR T cells with CD28 TM domains are better able to secrete cytokines than CAR T cells with CD8 TM domains (34). Based on cytokine secretion alone, it might be tempting to hypothesize that 4-1BB-CAR/CXCR5 T cells are less effective at viral control than CD28-CAR/CXCR5 T cells. However, our in vitro suppression and killing assay showed equal capability of both CD28- and 4-1BB-CAR/CXCR5 T cells to recognize and significantly suppress both SIV and HIV infection.

**FIGURE 6.** Decreased cytokine expression in human 4-1BB CAR T cells has no impact on HIV suppression or cytotoxicity in vitro. (A) Frequency of MBL+ cells producing indicated cytokine after overnight incubation with 8E5 cells, CD107a, and BFA (n = 6, multiple t test, *p < 0.05; ns, not significant). (B) Example staining of frequency of p24+HIV+ target cells after 48 h of coculture with either CD28-, 4-1BB-, or mock-transduced CAR T cells. 8E5 target cells were labeled with CTV and incubated for 48 h with mock-, hCD28-, or h4-1BB-CAR T cells. Percent p24 was determined by intracellular staining (n = 6, Wilcoxon matched-pairs rank test; ns, not significant). (C) Example staining of live target cells after 48 h of coculture. The frequency of live target cells was determined after 48 h of coculture with either mock-transduced, hCD28-, or h4-1BB-CAR/CXCR5 T cells (n = 6, Wilcoxon matched-pairs rank test; *p < 0.05. ns, not significant).
compared with mock-transduced cells. This finding is consistent with other studies showing similar in vitro cytotoxicity when comparing CD28- and 4-1BB-containing CAR T cells (29). As our studies were performed using total CD3\(^+\)-transduced cells, the relative contribution of CD8\(^+\) and CD8\(^+/0\) CAR T cells to virus suppression and target cell cytotoxicity is currently unknown. Future in vitro cytotoxicity and virus suppression assays combining different ratios of CD8\(^+\) and CD8\(^+/0\) CAR T cells might be informative about the relative contribution of CD8\(^+\) cells to virus suppression and control. Recent work suggests that both CD8\(^+\) and CD8\(^+/0\) CAR T cells can contribute to killing of virally infected cells (11).

Our in vitro cytotoxicity and suppression assays were designed to detect a dynamic range in frequency of infected cells in mock, CD28, and 4-1BB cultures to be able to distinguish a difference between CD8\(^+\) and 4-1BB-CAR/CXCR5 T cells while still showing activity of CAR/CXCR5 T cells compared with mock-transduced cells. Although many other CAR T cell killing assays use multiple increasing doses of CAR T cells over varying time points, our experiments were not focused on detecting maximal killing activity of CAR T cells. We used the ratio of 1:2 E:T cells because it gave us consistent results that differed significantly from mock cultures. In future experiments, it will be important to vary the E:T ratio and time of culture to detect suppression differences between 4-1BB- and CD28-CAR/CXCR5 T cells.

The level of apoptosis, as determined by annexin expression, showed species variation in experiments with our CD4 MBL CAR T cells. We observed that rhesus macaque 4-1BB-CAR T cells had a statistically significant reduction in annexin \(^+\) cells relative to the CD28-CAR T cells. However, human 4-1BB-CAR T cells showed a significant increase in annexin \(^+\) cells compared with the CD28-CAR T cells. The latter finding agrees with previous work using gammaretroviral vectors containing a human CD19 4-1BB-CAR (19, 20). Both the rhesus and human cells were transduced and expanded under identical conditions, so it is unlikely that there was an experimental difference leading to the opposing apoptotic responses. It is possible that human and rhesus cells have differing responses to the 4-1BB costimulatory domain of the CARs, and, if so, that difference might have implications for the application of primate models to development of human CAR T cell products. However, the number of test animals in this study was insufficient to make broad conclusions and further study, with a more robust sample size, is needed to confirm the impact of the costimulatory domain on apoptosis in rhesus macaques.

Ligand-independent tonic signaling has been reported in constructs containing both CD28 (21) and 4-1BB costimulatory domains (19). Our results are suggestive of tonic signaling in...
both rhesus macaque and human 4-1BB-CAR/CXCR5 T cells (measured by increased expression of CAR and CD95). We detected increased activation in 4-1BB- compared with CD28-CAR/CXCR5 T cells by D9 in rhesus macaque, whereas this increased activation did not occur until D15 in human PBMCs. However, we detected differential expansion of h4-1BB- and hCD28-CAR/CXCR5 T cells in short-term culture (D9), potentially due to increased apoptosis of h4-1BB-CAR/CXCR5 T cells. Importantly, whether tonic signaling is occurring in this system, it has no impact in vitro on virus suppression or cytotoxicity of our CAR T cells. Interpretation of our results for human PBMCs is limited by the small sample size, and the fold expansion might be skewed by two donors that had >30-fold expansion in the D9 culture. Importantly, note that previous studies of tonic signaling in vitro examined the phenomenon after triggering the CAR (19, 28), and we examined the same parameters after activation and expansion, with no CAR ligand. The focus on ligand-independent signaling in this study may explain some of the discrepancies between our findings and those presented in previous studies.

Overall, this study showed that replacing the CD28 costimulatory domain of CD4-MBL-CAR/CXCR5 T cells with a 4-1BB costimulatory domain induces functional CAR T cells, with capacity to suppress and kill virally infected cells in vitro. Due to the reported longevity of 4-1BB-CAR T cells in vivo, the 4-1BB costimulatory domain may be preferred for production of immunotherapeutic cells targeting HIV/SIV. However, in vivo studies are needed to compare efficacy and persistence of 4-1BB-CAR/CXCR5 T cells to CD28-CAR/CXCR5 T cells.

DISCLOSURES

P.J.S. is the co-founder and CSO of MarPam Pharma. The other authors have no financial conflicts of interest.

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REFERENCES


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### Supplemental Tables and Figures.

#### Supplemental Table 1. List of antibodies, fluorochromes, clones, and vendor used in all flow cytometry experiments.

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Supplemental Figure 1. Amino acid sequences of the Chimeric Antigen Receptors used in this study. (A.) Human CD4-MBL CD28 CAR. This sequence was previously published (6). (B.) Human CD4-MBL 4-1BB CAR. This sequence was adapted using human sequence from a patent for a 4-1BB CAR (24) and the CAR presented in A. (C.) Rhesus CD4-MBL CD28 CAR. This CAR was previously described (22). (D.) Rhesus CD4-MBL 4-1BB CAR. This sequence was adapted using rhesus specific sequences based on human sequence in a 4-1BB patent (24) and the rhesus CD4-MBL CAR.
Supplemental Figure 2. CXCR5 is functional, and expression is correlated with CD4-MBL in both CD28- and 4-1BB CAR/CXCR5-transduced cells. (A) Representative histogram plots showing MFI of CXCR5 in mock, CD28, and 4-1BB CAR/CXCR5-T cells. (B) Positive correlation between gMFI of CXCR5 and gMFI of MBL in both CD28- and 4-1BB CAR/CXCR5-T cells (n=10, Spearman correlation). (C) CXCR5 expression is associated with increased migration to CXCL13, with no impact on migration to CXCL12. Mock-, CD28-, and 4-1BB CAR/CXCR5-transduced cells were incubated at 37°C in Transwell plates with the indicated chemokine (CXCL13, pink; CXCL12, black). The percentage of cells migrating after 4 h was determined.
**Supplemental Figure 3.** (A) Example staining of live target cells after 48 h co-culture with CEMx174 cells (HIV-). (B) The frequency of live target cells was determined after 48 h co-culture with either mock transduced, hCD28- or h4-1BB-CAR/CXCR5-T cells (n=6, Wilcoxon matched-pairs rank test; ns=not significant).